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Characterization of three novel members of the zebrafish *Pax2/5/8* family: dependency of *Pax5* and *Pax8* expression on the *Pax2.1* (*noi*) function

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SUMMARY

The mammalian *Pax2*, *Pax5* and *Pax8* genes code for highly related transcription factors, which play important roles in embryonic development and organogenesis. Here we report the characterization of all members of the zebrafish *Pax2/5/8* family. These genes have arisen by duplications before or at the onset of vertebrate evolution. Due to an additional genome amplification in the fish lineage, the zebrafish contains two *Pax2* genes, the previously known *Pax[b]* gene (here renamed as *Pax2.1*) and a novel *Pax2.2* gene.

The zebrafish *Pax2.1* gene most closely resembles the mammalian *Pax2* gene in its expression pattern, as it is transcribed first in the midbrain-hindbrain boundary region, then in the optic stalk, otic system, pronephros and nephric ducts, and lastly in specific interneurons of the hindbrain and spinal cord. *Pax2.2* differs from *Pax2.1* by the absence of expression in the nephric system and by a delayed onset of transcription in other *Pax2.1* expession domains. *Pax8* is also expressed in the same domains as *Pax2.1*, but its transcription is already initiated during gastrulation in the primordia of the otic placode and pronephric anlage, thus identifying *Pax8* as the earliest developmental marker of these structures. The zebrafish *Pax5* gene, in contrast to its mouse orthologue, is transcribed in the otic system in addition to its prominent expression at the midbrain-hindbrain boundary.

The *no isthmus* (*noi*) mutation is known to inactivate the *Pax2.1* gene, thereby affecting the development of the midbrain-hindbrain boundary region, pronephric system, optic stalk and otic region. Although the different members of the *Pax2/5/8* family may potentially compensate for the loss of Pax2.1 function, we demonstrate here that only the expression of the *Pax2.2* gene remains unaffected in *noi* mutant embryos. The expression of *Pax5* and *Pax8* is either not initiated at the midbrain-hindbrain boundary or is later not maintained in other expression domains. Consequently, the *noi* mutation of zebrafish is equivalent to combined inactivation of the mouse *Pax2* and *Pax5* genes with regard to the loss of midbrain-hindbrain boundary development.

Key words: Pax, Zebrafish, *noi* mutation, CNS development, Isthmus, Eye, Ear, Kidney

INTRODUCTION

The nine mammalian *Pax* genes can be grouped into four distinct classes based on their similarity in sequence and expression (reviewed by Stuart et al., 1994). The subfamily consisting of *Pax2* (Dressler et al., 1990), *Pax5* (BSAP) (Adams et al., 1992) and *Pax8* (Plachov et al., 1990) encodes transcription factors that recognize their target genes via the highly conserved N-terminal paired domain (Czerny et al., 1993) and control gene transcription through a C-terminal regulatory module (Dörfler and Busslinger, 1996). All members of the *Pax2/5/8* family additionally share a conserved octapeptide motif and a partial homeodomain sequence (Czerny et al., 1997). These characteristic features are also present in the product of the zebrafish *Pax[b]* gene, which has been identified as the only member of the *Pax2/5/8* family in lower vertebrates (Krauss et al., 1991).

The *Pax2/5/8* genes are expressed in a spatially and temporally overlapping manner at the midbrain-hindbrain boundary and in the spinal cord of the mouse embryo (Nornes et al., 1990; Plachov et al., 1990; Adams et al., 1992). Outside of the CNS, the expression of *Pax2* and *Pax8* overlaps only partially in the developing excretory system (Dressler et al., 1990; Plachov et al., 1990). *Pax2* is, however, uniquely expressed during eye and ear morphogenesis (Nornes et al., 1990), *Pax5* throughout B-lymphopoiesis (Adams et al., 1992) and *Pax8* during thyroid gland development (Plachov et al., 1990). The zebrafish *Pax[b]* gene is also transcribed at the midbrain-hindbrain junction, in the caudal CNS, in the developing eye and ear as well as during kidney morphogenesis, thus indicating that *Pax[b]* most closely resembles the mouse *Pax2* gene in its embryonic expression pattern (Krauss et al., 1991).

Consistent with these expression profiles, targeted gene inactivation has revealed essential roles for the murine *Pax2/5/8* genes during CNS development and organogenesis. The loss of *Pax2* leads to multiple defects in tissues where this gene is uniquely expressed. Thus, mice lacking *Pax2* fail to develop an excretory system, exhibit pathfinding defects of the optic nerve and show abnormal patterning of the inner ear (Torres et al., 1995, 1996; Favor et al., 1996). In *Pax5* mutant mice, B cell development is arrested at an early precursor stage (Urbánek et al., 1994; Nutt et al., 1997), while mice homozygous for a *Pax8* mutation suffer from hypoplasia of the thyroid gland (Mansouri et al., 1998). Mice carrying a single mutation in *Pax2* or *Pax5* exhibit, however, only a mild, variable and strain background-dependent phenotype in the developing midbrain and cerebellum, i.e. in those CNS structures that are derived from the common expression domain of these two genes at the midbrain-hindbrain junction (Urbánek et al., 1994; Torres et al., 1996). Grafting experiments furthermore demonstrated that the midbrain-hindbrain boundary tissue functions as an organizing center in patterning of the midbrain and cerebellum (reviewed by Wassef and Joyner, 1997). This embryonic CNS region is, however, consistently lost in *Pax2*, *Pax5* double-mutant embryos, thus indicating that the two genes cooperate in midbrain and cerebellum development (Urbánek et al., 1997; Schwarz et al., 1997).

The formation of the midbrain-hindbrain boundary (known as the isthmus) is prevented in the zebrafish embryo by injection of neutralizing anti-Pax[b] antibodies (Krauss et al., 1992). Moreover, several alleles of *Pax[b]* have recently been isolated in a genetic screen for zebrafish developmental mutants (Brand et al., 1996). These *noi* (*no isthmus*) mutations interfere with development of the midbrain-hindbrain boundary region in homozygous embryos, thus indicating that the loss of *Pax[b]* function is sufficient to prevent normal functioning of the organizing center at the isthmus (Brand et al., 1996; Lun and Brand, 1998). In addition, the morphogenesis of the pronephros is severely affected, the size of the otic vesicle is reduced, and pathfinding errors of the optic nerve are observed in *noi* mutant embryos (Brand et al., 1996; Macdonald et al., 1997). Hence, the zebrafish *noi* and mouse *Pax2* mutations resemble each other in phenotype, with the notable exception that *Pax5* expression is able to compensate in the mouse embryo for the loss of *Pax2* at the midbrain-hindbrain boundary. This discrepancy in phenotype has raised the possibility that the zebrafish contains only a single member of the *Pax2/5/8* family, *Pax[b]* (Brand et al., 1996).

Here we report the cloning and characterization of the full complement of zebrafish *Pax2/5/8* genes, which revealed a strict dependency of *Pax5* and *Pax8* expression on the *noi* function at the midbrain-hindbrain boundary. This genetic interaction offers an explanation why a single mutation in the zebrafish *Pax[b]* gene is functionally equivalent to the double mutation of the mouse *Pax2* and *Pax5* genes.

MATERIALS AND METHODS

Cloning of *Fugu Pax* genes

A *Fugu rubripes* cosmid library, kindly supplied by Carola Burgtorf (Max-Planck-Institut für Molekulare Genetik, Berlin), was screened at low stringency with a mixed probe consisting of the coding sequences of *zPax[b]* (Krauss et al., 1991), *mPax2* (Dressler et al., 1990), *hPax5* (Adams et al., 1992) and *hPax8* cDNA (Kozmik et al., 1993). The cosmids ICRFc66C0388Q1.2 and ICRFc66M1812Q1.2 were subsequently shown by PCR analysis to contain the *Pax5* and *Pax8* genes, respectively. Exon-containing DNA fragments were subcloned, and the amino acid sequences were assembled from the different exons.

Oligonucleotides

The following oligonucleotides were used:

(1) 5′-GCGGAATTCGGN(A/T)(G/C)NAAPuCCNAAPuGTNGC-3′
(2) 5′-GCGAGAAGCTTATPyTCCCANGCPuAACATNGT-3′
(3) 5′-GCGGAATTCGTNAAPyCAPuPyTNGGNGGNGTNTT-3′.

Pax2.1:

5′-GCAGCCTTTCCACCCATCCTC-3′ (δ)
5′-GCTCTGGCTTGATGTGTTCTG-3′ (δ).

Pax2.2:

5′-TTGTCTTTTGTAGTCCGCTA-3′ (α)
5′-CTTTCTAGACCTTTACCTGACGTCGTGCG-3′ (β)
5′-CTTCTCGAGPyPyAPuTGNC(G/T)PuTCPuTANGC-3′ (γ)
5′-ACCGTTTCACCCTTCACCAGA-3′ (δ)
5′-CGTCAGGAAAGGCGGGTCTCT-3′ (δ).

Pax5:

5′-TTTGGCGTTTGTAATCAGCA-3′ (α)
5′-CCCACTAGTTGTTGACAAAATTGCTGAT-5′ (β)
5′-TCAGGGTCAPuTGNC(G/T)PuTCPuTA-3′ (γ)
5′-GTGGCAGTGACGCAGGTTTCT-3′ (δ)
5′-GCTGTTCTTCATCTCCTCCAA-3′ (δ).

Pax8:

5′-GCTTGTACTCCGCGATCTTC-3′ (α)
5′-AGGTCTAGAGGAGGTGATCCGGCAAAGGA-3′ (β)
5′-ACTANAGPuTGPuTCPu(A/T)ANGC-3′ (γ)
5′-GCTCCGCCGTCACTCCTCCTG-3′ (δ)
5′-GCTGTCGCTGCTGTAGTGTCT-3′ (δ).

Cloning of zebrafish *Pax2/5/8* cDNA

DNA fragments of the zebrafish *Pax2/5/8* genes were isolated by PCR from genomic DNA with the oligonucleotide pair 1/2 derived from exon 3 of mammalian *Pax2/5/8* genes. Four different PCR products were cloned at similar ratios and sequenced. Gene-specific antisense oligonucleotides (set α) were designed from these sequences and used in combination with the degenerate sense oligonucleotide 3 (located at the 5′ end of exon 2) to perform RT-PCR on RNA isolated from 9- to 24-hour zebrafish embryos. RT-PCR amplification from embryonic RNA was next performed with gene-specific sense oligonucleotides (set β), located 3′ of primer 3 in exon 2, and degenerate antisense oligonucleotides, the design of which was based on the conservation of C-terminal sequences between the pufferfish and mammalian orthologues (set γ). Only the most abundant product of each PCR reaction was cloned and sequenced. Full-length *Pax2.2* cDNA clones were isolated by screening a 1-day-old embryo cDNA library (Stratagene) with a *Pax2.2* PCR probe.

Quantitative RT-PCR assay

Gene-specific primers (set δ) derived from different exons of the zebrafish *Pax2/5/8* genes were used for RT-PCR assay as described (Kelly et al., 1995). The number of PCR cycles (25-30) was empirically determined for each primer pair to ensure amplification within the linear range. Primers of the housekeeping gene *max* (Kelly et al., 1995) were used for standardization of the reverse-transcribed cDNA input.

Whole-mount in situ hybridization

Embryos of the zebrafish (*Danio rerio*) were raised at 28.5°C and

staged according to Kimmel et al. (1995). Whole-mount in situ hybridization was essentially performed as described (Hauptmann and Gerster, 1994), using single-stranded RNA probes labelled with digoxygenin-UTP (Boehringer-Mannheim). The *Pax2.1* probe contained 609 nucleotides of 3′ non-coding sequences and 45 base pairs of exon 10 sequences up to the *Sma*I site (Krauss et al., 1991). The *Pax2.2* probe consisted of 970 base pairs of 3′ non-coding sequence up to the *Pvu*II site. The 3′ non-coding sequences of the *Pax2* genes do not cross-hybridize, due to the lack of sequence homology. The *Pax5* probe consisted of 770 base pairs spanning the coding sequences from the start of exon 4 to the stop codon. The *Pax8* probe was composed of 798 base pairs extending from exon 5 to exon 9. The zebrafish *eng3* (Ekker et al., 1992) and mouse *Pax8* (Adams et al., 1992) probes were previously described. Hybridizations and washes were performed at 58-60°C.

Accession numbers

The sequences of the *zPax2.2*, *zPax5* and *zPax8* cDNA as well as the *fPax5* and *fPax8* exons have been submitted to GenBank (accession numbers AF072547-AF072556).

RESULTS

The fish genome contains four members of the *Pax2/5/8* family

Three different cosmids were isolated by screening a genomic library of the Japanese pufferfish, *Fugu rubripes*, with a mixed probe consisting of zebrafish *Pax[b]*, mouse *Pax2*, human *Pax5* and human *Pax8* cDNA. The gene present on one clone proved to be highly similar to the zebrafish *Pax[b]* gene (Krauss et al., 1991) and was therefore not further analysed. The two other cosmids contained novel members of the *Pax2/5/8* family. Comparison of the amino acid sequences encoded by the different exons of these cosmids (Fig. 1A) identified the two genes as fish homologues of *Pax5* and *Pax8*. Hence, the fish genome also possesses all three members of the *Pax2/5/8* family.

To study the developmental expression of the novel *Pax* genes, we cloned the corresponding cDNA sequences from zebrafish. The different *Pax* cDNAs were isolated by PCR amplification in two steps. First, the paired domain exons were amplified from genomic DNA with *Pax2/5/8*-specific primers (Czerny et al., 1997) and sequenced. Next, gene-specific primers derived from these paired domain sequences were used, in combination with degenerate primers deduced from the C-terminal sequences of the respective *Fugu* and mammalian *Pax* genes, for RT-PCR amplification of the entire coding sequences from embryonic RNA. Surprisingly, we isolated four different cDNA sequences coding for zebrafish Pax5, Pax8 and two closely related Pax2 proteins (Fig. 1A). One of the two *Pax2* cDNAs corresponded to the known *Pax[b]* transcript (Krauss et al., 1991), while the coding sequence of the second gene was 80% identical with *Pax[b]* at the nucleotide level. Most of the sequence changes (86%) observed between the two *Pax2* cDNAs occurred in silent codon positions consistent with the high amino acid sequence conservation (93% identity; Fig. 1). Comparison with the mouse Pax2 protein unequivocally identified the two zebrafish genes as homologues of the mammalian *Pax2* gene (Fig. 1). Hence, we renamed the zebrafish *Pax[b]* gene as *Pax2.1* and refer to the second *Pax2* homologue as *Pax2.2*. To exclude possible PCR artefacts, we isolated full-length *Pax2.2* cDNA from an embryonic cDNA library. Sequence analysis confirmed that the *Pax2.2* mRNA contains a contiguous open reading frame coding for a functional Pax2 protein (Fig. 1A). Interestingly however, the two *Pax2* genes differ almost completely in their 5′ and 3′ non-coding sequences, in marked contrast to the conservation of their coding regions. Several other gene families have previously been shown to contain more members in zebrafish compared to mammals, indicating that a partial genome duplication has occurred in the zebrafish lineage (Postlethwait et al., 1998). Hence, the two zebrafish *Pax2* genes also appear to originate from such a genome amplification.

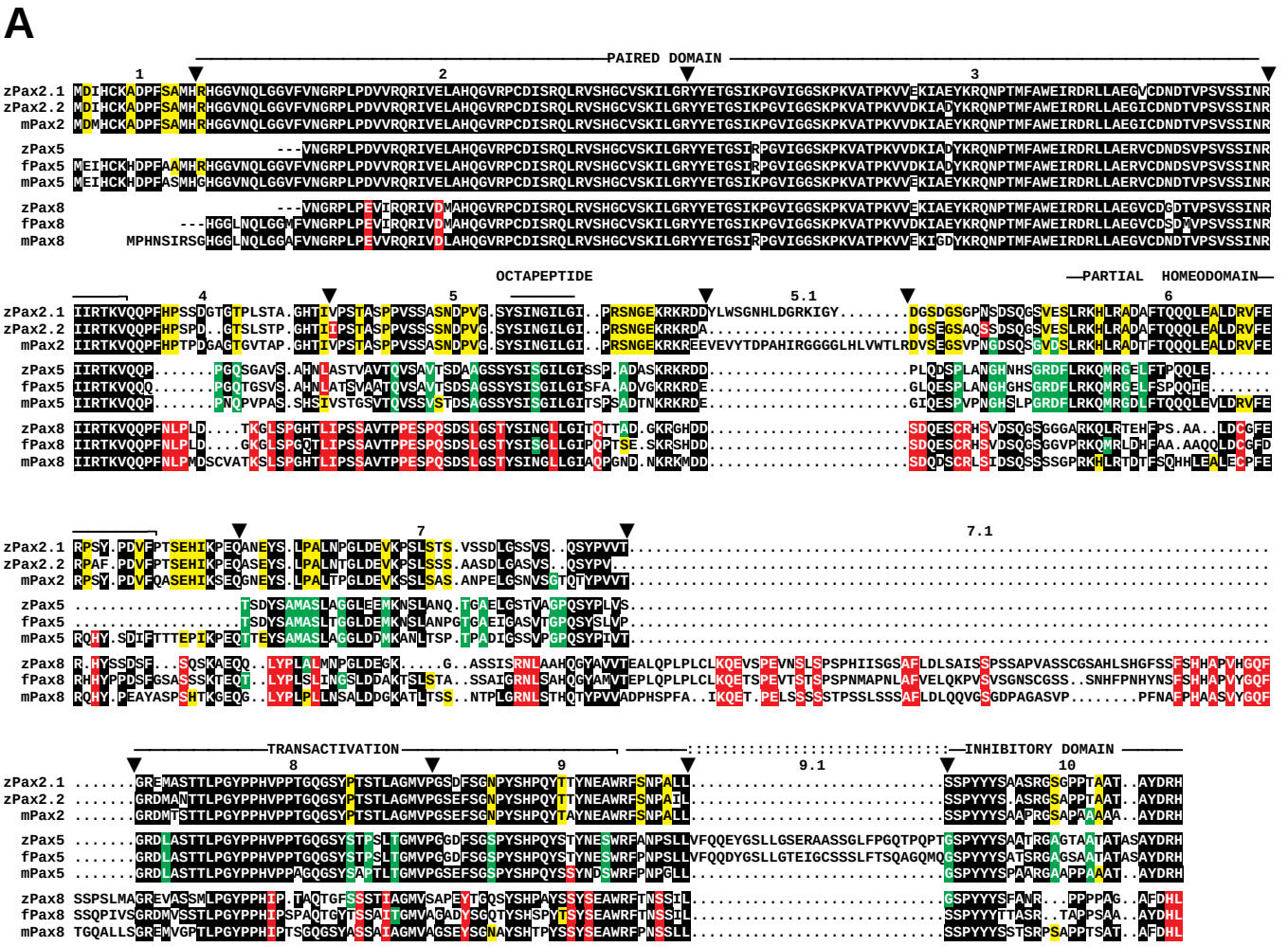
Evolution of the fish Pax2/5/8 proteins

All three proteins of the vertebrate Pax2/5/8 family share a high degree of sequence identity, as shown by the alignments in Fig. 1A. Outside the conserved sequence blocks each Pax protein is, however, characterized by unique amino acid sequences that have been conserved between fish and mammals, thus allowing an unequivocal assignment of the cloned genes as fish orthologues of *Pax2*, *Pax5* and *Pax8*, respectively (Fig. 1A). Quantitative analysis of the sequence differences resulted in a phylogenetic tree of the vertebrate Pax2/5/8 proteins (Fig. 1B), which is in agreement with the known divergence of fish and mammals. Zebrafish and *Fugu* are both euteleosts sharing a common ancestor approx. 120 million years ago, while fish and mammals diverged approx. 440 million years ago (Benton, 1997). Interestingly, the Pax2 proteins have diverged the least (12%) and the Pax8 proteins the most (25%) between fish and mammals, while the Pax5 proteins show an intermediate divergence of 19%. Consistent with this observation, the Pax8 proteins of zebrafish and *Fugu* diverged twice as fast as the respective Pax5 proteins (18% versus 9%; Fig. 1B), although all four genes evolved at a similar rate by silent substitutions. We conclude therefore that the different members of the *Pax2/5/8* gene family have been subjected to different selection pressures during vertebrate evolution. The rate of divergence (Pax8>Pax5>Pax2) suggests that Pax2 most closely resembles the ancestral gene that gave rise to the three members of the *Pax2/5/8* family by gene duplications before or at the onset of vertebrate evolution.

The vertebrate Pax2/5/8 proteins are highly conserved in those domains that have been assigned a specific function. Hence, the N-terminal paired domain involved in DNA binding (Czerny et al., 1993), the octapeptide region implicated in protein-protein interactions, and the C-terminal regulatory module consisting of activating and inhibitory sequences (Dörfler and Busslinger, 1996) are almost invariant among the different Pax2/5/8 transcription factors (Fig. 1A). In addition, a region with sequence similarity to the N-terminal part of the homeodomain was previously identified in Pax2/5/8 proteins (Krauss et al., 1991). This homeodomain homology region is, however, less well conserved and even partially deleted in the fish Pax5 proteins, suggesting that it may be less important for the function of these transcription factors.

Comparison of the exon-intron structure of the *Fugu* and mammalian *Pax* genes revealed that intron positions have been strictly conserved among members of the vertebrate *Pax2/5/8* family, with one exception (Fig. 1A). Exon 6 of the fish *Pax5* genes is shorter, due to the use of an intron junction that is present only as a cryptic splice site within exon 6 of mammalian *Pax5* genes (Adams et al., 1992). Moreover, the *Pax2* and *Pax8* transcripts are known to be subject to extensive

alternative splicing, which results in the inclusion of additional exons (Kozmik et al., 1993; Heller and Brändli, 1997; Lun and Brand, 1998). Exons that are present in all *Pax2/5/8* family members have been numbered from 1 to 10 in Fig. 1A, whereas the suffix '.1' refers to the less well conserved, non-canonical exons of the different *Pax* genes. The zebrafish *Pax2.1* and mouse *Pax2* genes contain the alternative exon 5.1, which codes for a divergent amino acid sequence of variable length



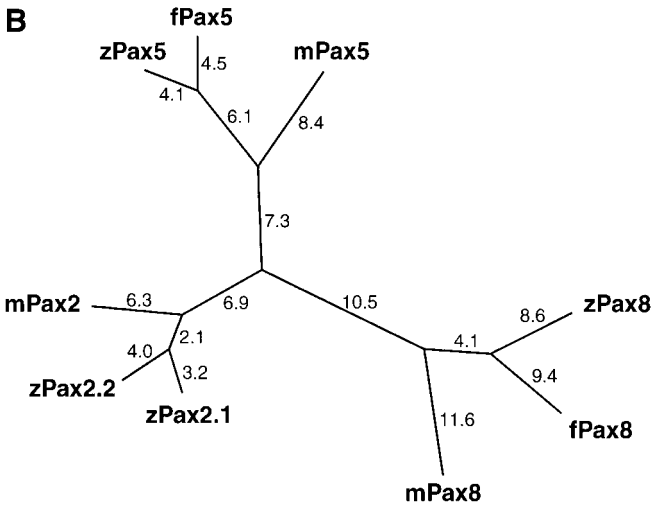


Fig. 1. Sequence comparison and phylogenetic relationship of fish and mouse Pax2/5/8 proteins. (A) Sequence alignment. The Pax2, Pax5 and Pax8 proteins of fish are compared with their mouse homologues (Dressler et al., 1990; Plachov et al., 1990; Adams et al., 1992). The Pax2.1 protein was previously referred to as Pax[b] (Krauss et al., 1991). The amino acid sequence of the zPax2.2 protein was deduced from full-length cDNA, while the zPax5 and zPax8 sequences were derived from cDNAs amplified by RT-PCR from embryonic RNA. The amino acid sequences of the *Fugu* Pax5 and Pax8 proteins were assembled from exon sequences identified on the respective cosmids. Amino acids shared by at least five proteins are highlighted by black overlay. A colour code denotes those residues that are characteristic of individual Pax proteins. Dots indicate the gaps introduced for optimal sequence alignment and dashes the missing N-terminal sequences. Exons are numbered, and arrowheads indicate intron positions that have been conserved between the cloned *Fugu* and mammalian *Pax* genes (Kozmik et al., 1993; Busslinger et al., 1996). Only the Pax2a and Pax8a isoforms of the mouse are shown. (B) Evolutionary relationship of vertebrate Pax2/5/8 proteins. The sequences of only the common exons 1-10 were used for calculating the phylogenetic tree by the GCG program Clustree. The length of each branch is proportional to the indicated percentage sequence divergence from the branchpoint.

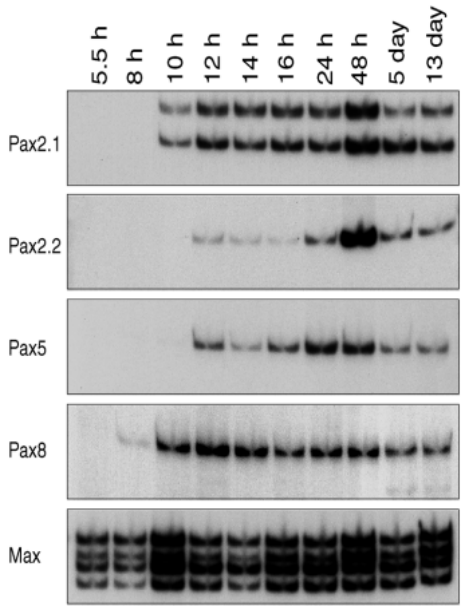


Fig. 2. Temporal expression of the *Pax2/5/8* genes during zebrafish embryogenesis. Total RNA of the indicated developmental stages was analyzed by quantitative RT-PCR using gene-specific primers. All cDNA sequences were amplified across the exon 5-exon 6 junction. The two PCR products of the *Pax2.1* mRNA differ by the presence (upper band) or absence (lower band) of exon 5.1. The ubiquitiously expressed mRNA of the *max* gene (Kelly et al., 1995) was analyzed to quantitate the reverse-transcribed cDNA input of the PCR reaction.

(Fig. 1A). As shown by quantitative RT-PCR analysis, the zebrafish *Pax2.1* transcripts containing or lacking this additional exon are generated at a similar ratio throughout development (Fig. 2). A similar insertion could, however, not be detected in the *Pax2.2*, *Pax5* and *Pax8* transcripts (Fig. 2), suggesting that exon 5.1 is unique to the *Pax2(.1)* gene. Moreover, the alternative exon 7.1 coding for 69-80 amino acids has so far only been found in *Pax8* genes of fish and mammals (Fig. 1A). Of particular interest is an in-frame insertion of 29 amino acids in the inhibitory domain of the fish Pax5 proteins. The additional exon 9.1 was present not only in the *Fugu Pax5* gene, but also in the cloned zebrafish *Pax5* transcripts, suggesting that the function of the C-terminal inhibitory domain is controlled by alternative splicing.

Pax gene expression at the midbrain-hindbrain boundary of the fish embryo

Quantitative RT-PCR analyses with RNA of different developmental stages (Fig. 2) demonstrated that the *Pax8* mRNA was first detected at 8 hours postfertilization (75% epiboly). At this stage, *Pax2.1* expression was barely visible, but then strongly increased until 10 hours of embryogenesis (bud stage). Subsequently, the expression of *Pax2.2* and *Pax5* was observed at 12 hours of development (5 somites). Interestingly, the expression of all four *Pax* genes was maintained for at least 13 days postfertilization.

We next studied the spatial expression pattern of all four *Pax* genes by whole-mount in situ hybridization with gene-specific probes that consisted either of C-terminal coding (*Pax5* and *Pax8*) or 3′ non-coding (*Pax2.1* and *Pax2.2*) sequences. The expression of *Pax2.1* was previously reported to initiate between 8 and 9 hours postfertilization in two lateral stripes of the anterior neural plate, which mark the area of the prospective midbrain-hindbrain border (Krauss et al., 1991; Kelly and Moon, 1995; see also Fig. 3A). At this time, no transcripts of the other three *Pax* genes could yet be detected in the same region of the CNS. *Pax5* expression was first observed at the 4-5-somite stage (Fig. 3C) followed by weak *Pax2.2* expression in the 5-somite embryo (Fig. 3B). Later, *Pax8* expression was initiated at the midbrain-hindbrain boundary between the 7- and 10-somite stage (Fig. 3D). The expression of all four *Pax* genes was maintained in this brain region at a high level throughout the segmentation period (10- and 20-somite stages; Figs 4, 5 and 6D). In 1-day-old embryos, the midbrain primordium has been subdivided to form the dorsal optic tectum and the ventral

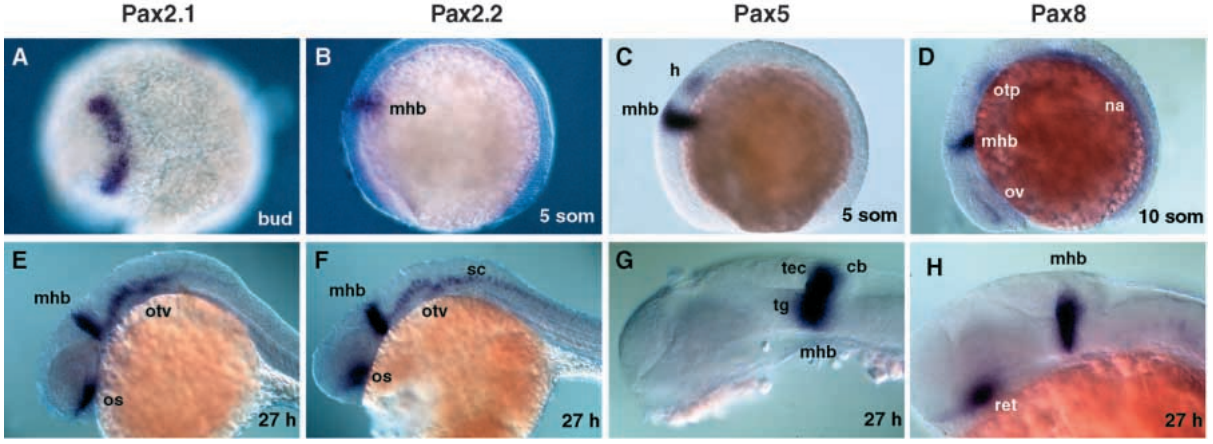


Fig. 3. Expression of *Pax2/5/8* genes at the midbrain-hindbrain boundary of zebrafish embryos. Transcripts of *Pax2.1* (A,E), *Pax2.2* (B,F), *Pax5* (C,G) and *Pax8* (D,H) were detected by whole-mount in situ hybridization in embryos from 10 hours (bud stage) to 27 hours postfertilization. Embryos are shown in lateral view (B-H) except for the bud stage (A) which is a dorsal view. cb, cerebellum; h, hindbrain; mhb, midbrain-hindbrain boundary; na, (pro)nephric anlage; nd, nephric duct; pn, pronephros; os, optic stalk; otp, otic placode; otv, otic vesicle; ov, optic vesicle; ret, retina; som, somite; sc, spinal cord; tec, tectum; tg, tegmentum.

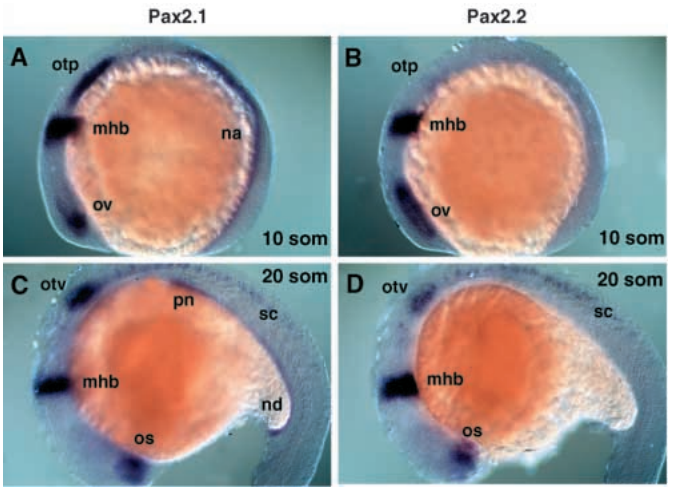


Fig. 4. Comparison of the *Pax2.1* and *Pax2.2* expression patterns. The expression of *Pax2.1* (A,C) and *Pax2.2* (B,D) was analyzed by whole-mount in situ hybridization in 10- and 20-somite embryos (shown in lateral view). For abbreviations see legend to Fig. 3.

tegmentum, which converge posteriorly to join at the midbrain-hindbrain constriction (isthmus). It is exactly this region representing the posterior mesencephalon and isthmus that expresses all four *Pax2/5/8* genes (Fig. 3E-H). *Pax* gene transcripts were, however, not detected in the floor and roof plates. Moreover, the *Pax2.1*, *Pax2.2* or *Pax8* mRNAs are absent in the dorsal region immediately posterior to the midbrain-hindbrain constriction, which represents the primordium of the cerebellum. In contrast, the *Pax5* expression domain is broader at the pharyngula stage, as it extends into the anterior hindbrain territory and the tegmental region of the midbrain (compare Fig. 3G with 3E,F,H). In summary, all members of the *Pax2/5/8* subclass are expressed at the midbrain-hindbrain junction. However, the onset of expression differs, with *Pax2.1* being expressed first (before somitogenesis) followed by *Pax5* (at 4 somites), *Pax2.2* (at 5 somites) and lastly by *Pax8* (at approx. 9 somites).

Pax2.2 is transcribed in a subset of the *Pax2.1* expression domains

The *Pax2.1* gene is known to be expressed, in addition to the midbrain-hindbrain boundary, in four other regions of the embryo, i.e. in the optic stalk, the otic placode and vesicle, in specific neurons of the hindbrain and spinal cord as well as in the pronephric anlage and ducts (Krauss et al., 1991; Mikkola et al., 1992; Püschel et al., 1992; see Fig. 4A,C). As shown in Fig. 4B and D, the *Pax2.2* gene is also transcribed in all *Pax2.1* expression domains, with one exception. The *Pax2.2* gene is not expressed at any stage of pronephros development. Moreover, *Pax2.2* differs from *Pax2.1* in other expression domains, with regard to its temporal onset and transcript levels.

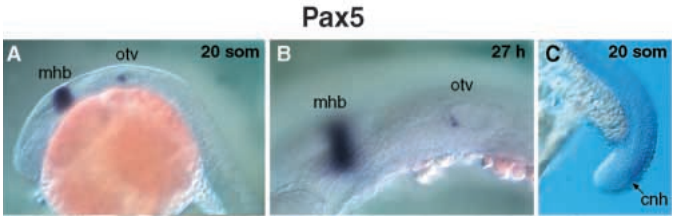


Fig. 5. *Pax5* expression in the otic vesicle and chordoneural hinge region. Whole-mount in situ hybridization of 20-somite (A) and 27-hour (B) embryos identified *Pax5*-expressing cells in the inner cell layer of the anterior otic vesicle (otv). At the 20-somite stage, weak Pax5 staining is also seen in the chordoneural hinge (cnh) region of the tail (C).

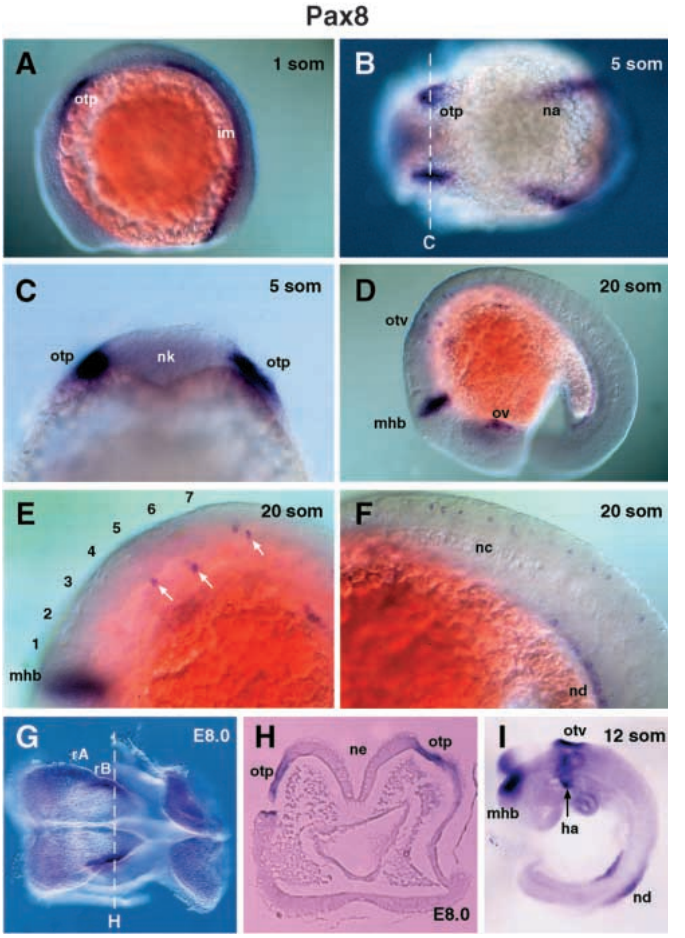


Fig. 6. Identification of novel expression domains of the vertebrate *Pax8* gene. (A) Early *Pax8* expression in the intermediate mesoderm (im) and in the anlage of the otic placode (otp). (B) Dorsal view of a 5-somite embryo with lateral expression of *Pax8* in the otic placode and pronephric anlage (na). (C) Optical cross-section of the 5-somite embryo at the level of the otic placode (as indicated in B), which localizes the Pax8 staining in the ectoderm adjacent to the neural keel (nk). Lateral view (D) and higher magnifications (E,F) of a 20-somite embryo. *Pax8* expression is no longer observed in the otic vesicle (otv), while it is maintained in the pronephros (out of focal plane) and nephric duct (nd). Arrows in E indicate individual *Pax8*-expressing neurons in the hindbrain, and numbers refer to individual rhombomeres. (G) Dorsal view of an 8.0-day mouse embryo (before somitogenesis) hybridized with a mouse *Pax8* RNA probe. (H) Cross-section through the same embryo at the level indicated in G. Pax8 staining is detected lateral to the neuroepithelium (ne) of rhombomere B (rB) in the ectodermal layer corresponding to the otic placode. (I) Mouse *Pax8* expression at the 12-somite stage. ha, hyoid arch; nc, notocord; ov, optic vesicle.

As in the midbrain-hindbrain boundary region, the expression of *Pax2.2* is delayed relative to that of *Pax2.1* in the optic stalk (5-7 somites versus 3 somites) and otic placode (6 somites versus 3 somites) (Figs 4, 7A; data not shown). The expression of both genes is, however, coordinately activated at around the 12-somite stage in the hindbrain and spinal cord (Fig. 4C,D; data not shown). Later in embryogenesis, the two *Pax2* genes are similarly transcribed in the common expression domains, except in the developing ear where their relative mRNA levels vastly differ (Fig. 4). While the *Pax2.1* gene is expressed in the otic placode (10 somites) and vesicle (20 somites) at a similar level to the midbrain-hindbrain boundary, the expression of the *Pax2.2* gene is considerably weaker in the otic region compared to the midbrain-hindbrain junction (Fig. 4). Hence apart from the developing pronephros, the *Pax2.2* gene is expressed in the same regions of the embryo as *Pax2.1*, albeit with a delay of approx. 2 hours (except in the hindbrain and spinal cord) and at a significantly lower level in the otic region.

Pax5 expression in the developing ear

In contrast to its prominent expression at the midbrain-hindbrain boundary, the *Pax5* gene is transiently and weakly expressed in a transverse stripe of the hindbrain from the 5- to 10-somite stages (Fig. 3C, data not shown). Later, *Pax5* expression is observed in the anterior part of the otic vesicle from about the 17-somite stage onwards (Figs 5A,B and 8C). This region constitutes a subset of the *Pax2.1* expression domain, which extends along the entire otic vesicle (Fig. 4C). At 20 somites, weak *Pax5* expression is also detected at the posterior end of the spinal cord in the chordoneural hinge region (Fig. 5C). In this context it is interesting to note that the mouse *Pax5* gene is not transcribed in the otic region, whereas it is weakly expressed in the hindbrain and along the spinal cord (Adams et al., 1992; Asano and Gruss, 1992; Urbánek et al., 1994). Hence, the expression of *Pax5* in the otic system has not been conserved during vertebrate evolution.

Early expression of *Pax8* in the otic placode and pronephric anlage

Pax8 is the first gene of the *Pax2/5/8* family, which is highly transcribed already at 80% epiboly in two separate domains. The anterior domain occupies a position posterior to the prospective midbrain-hindbrain boundary (Fig. 6A,B) and expresses *Pax8* in the ectoderm on both sides of the neural keel, as shown by optical and paraffin cross-sections (Fig. 6C, data not shown). This expression domain corresponds to the primordium of the otic placode, which is first visible as a thickening of the ectodermal cell layer at about the 3-somite stage. At this time, expression of the *Pax2.1* gene is also initiated in the otic placode (Kelly and Moon, 1995; see above), while transcription of the *Pax8* gene is down-regulated soon thereafter. At the 10-somite stage, *Pax8* transcripts are only weakly expressed (Fig. 3D) and are no longer visible in the otic vesicle of the 20-somite embryo (Fig. 6D), indicating that the *Pax8* gene is only transiently expressed during early development of the otic region. Due to its transient nature, *Pax8* expression has not yet been reported in the otic placode of the mouse embryo (Plachov et al., 1990; Asano and Gruss, 1992). Indeed, re-examination of presomitic 8.0-day mouse embryos revealed *Pax8* expression at the level of rhombomere B on both sides of the neural plate where the otic placode is formed (Fig. 6G,H). At 12 somites, *Pax8* expression was still observed in the otic vesicle (Fig. 6I), but thereafter was lost in this organ of the mouse embryo (data not shown). Hence, *Pax8* represents the earliest known marker for the development of the otic system both in fish and mammals.

The posterior *Pax8* expression domain extends in early embryos from the middle of the anteroposterior axis to its caudal end all along the edges of the embryonic shield (Fig. 6A,B). Within this domain, *Pax8* expression was localized on cross-sections to the intermediate mesoderm (data not shown). In contrast to the transient expression in the otic placode, *Pax8* transcription is maintained in the intermediate mesoderm of 10-somite embryos (Fig. 3D) and is later seen in the pronephros as well as in the nephric ducts of 20-somite and 27-hour embryos (Figs 6F, 9E). In summary, *Pax8* expression is similar to that of *Pax2.1* in the developing excretory system,

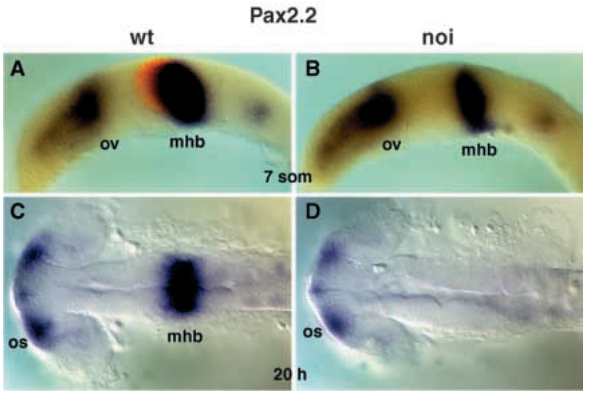


Fig. 7. *Pax2.2* expression is independent of the *noi* (*Pax2.1*) function. Transcripts of the *Pax2.2* gene were analysed by whole-mount in situ hybridization in wild-type (A,C) and *noi*^{tu29a} mutant (B,D) embryos at 7 somites and 20 hours postfertilization. The 7-somite embryos (A,B; lateral view) were genotyped by double staining with *Pax2.2* (blue) and *eng3* (red) RNA probes. Homozygous *noi* mutant embryos were identified by the absence of *engrailed* (*eng*) expression at the midbrain-hindbrain boundary (mhb) (Brand et al., 1996). At 20 hours, the mhb tissue is entirely lost in *noi* mutant embryos (dorsal view).

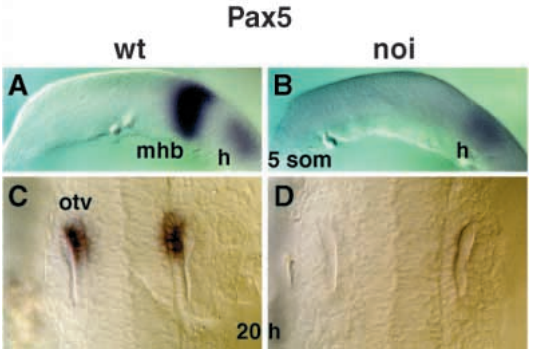


Fig. 8. Dependency of *Pax5* expression on the *noi* (*Pax2.1*) function. *Pax5* expression was analyzed in wild-type (A,C) and *noi*^{tu29a} mutant (B,D) embryos at the 5-somite stage (lateral view) and 20 hours postfertilization (enlarged dorsal view, anterior to the top). For abbreviations see legend to Fig. 3.

except that it is initiated much earlier in the precursor cells of the pronephric anlage.

The *Pax8* gene is furthermore transcribed in four areas of the central nervous system, including the midbrain-hindbrain boundary region described above. Secondly, in contrast to the mouse (Asano and Gruss, 1992), *Pax8* expression is also detected in the optic vesicle of zebrafish embryos from the 9-10-somite stage onwards (Figs 3D, 6D, 9A) and is later confined to the retina in pharyngula embryos (Figs 3H, 9C). The hindbrain is the third CNS domain, where three sets of neurons express *Pax8* on either side of the midline in rhombomeres 5, 6 and 7 of the 20-somite embryo (Fig. 6E). The fourth expression domain becomes visible in the anterior half of the spinal cord at the 20-somite stage and encompasses individual well-spaced cells, which can be classified as interneurons on the basis of their location along the dorsoventral axis of the neural tube (Fig. 6D,F). In conclusion, the *Pax8* gene of zebrafish is transcribed in all *Pax2.1* expression domains, although the temporal regulation of the two genes differs substantially in these regions.

The *Pax2.1* (*noi*) function is essential for initiation of *Pax5* and *Pax8* expression at the midbrain-hindbrain boundary

One important conclusion of the above expression analysis is the high conservation of the temporal onset and expression pattern of *Pax2*, *Pax5* and *Pax8* at the midbrain-hindbrain boundary between zebrafish (Fig. 3) and mouse (Adams et al., 1992; Asano and Gruss, 1992; Rowitch and McMahon, 1995). Nevertheless, genetic evidence has suggested distinct roles for these genes in the two species. In the mouse, *Pax2* and *Pax5* can functionally compensate for each other, as development of the midbrain-hindbrain boundary region is lost only in compound *Pax2*, *Pax5* mutant embryos (Urbánek et al., 1997; Schwarz et al., 1997). In contrast, mutation of only one of four zebrafish genes, *Pax2.1* (*noi*), leads to loss of the isthmic region and most of the midbrain (Brand et al., 1996). To address this paradoxical issue, we have examined the expression pattern of the novel zebrafish *Pax2/5/8* genes in *noi* (*Pax2.1*) mutant embryos. For this purpose, we have used the noi^{tu29a} null allele (Brand et al., 1996), which carries a nonsense mutation in the paired box of *Pax2.1*, thus resulting in a truncated protein lacking any DNA-binding and transactivation function (Lun and Brand, 1998). Homozygous *noi* mutant embryos, which fail to express *engrailed* genes at the midbrain-hindbrain boundary (Brand et al., 1996), were identified at the 7-somite stage by double staining with *Pax2.2* and *eng3* probes. As shown in Fig. 7B, the *Pax2.2* gene is transcribed at the midbrain-hindbrain boundary of these mutant embryos, indicating that the isthmic region initially develops even in the absence of *Pax2.1* (*noi*) function, consistent with previous observations (Brand et al., 1996). A partial loss or respecification of the isthmic tissue is, however, already evident at this stage, as the *Pax2.2* expression domain in *noi* mutants (Fig. 7B) is only half as wide as in control embryos (Fig. 7A). At 20 hours of development, the entire isthmus is deleted (Brand et al., 1996), which entails the loss of *Pax2.2* expression in mutant embryos (Fig. 7D).

In contrast to *Pax2.2*, no trace of *Pax5* expression could be detected in the midbrain-hindbrain boundary region of *noi* mutant embryos even at the 5-somite stage, although *Pax5* expression was properly initiated in the hindbrain (Fig. 8B). Likewise, *Pax8* expression was never observed at the midbrain-hindbrain boundary, yet was normal in the otic placode and pronephric anlage of mutant embryos at the 9-somite stage (Fig. 9B). The isthmic tissue is still present in these early embryos, as indicated by *Pax2.2* expression (Fig. 7B), and hence these data indicate that a functional Pax2.1 (Noi) protein is required for transcriptional activation of *Pax5* and *Pax8* at the midbrain-hindbrain boundary in contrast to *Pax2.2*. Importantly, the *Pax2.1* (*noi*) mutation interferes with *Pax5* expression in the fish and can thus be regarded as formally equivalent to combined inactivation of *Pax2* and *Pax5* in the mouse. These data therefore offer an explanation for why different genetic alterations in the two species result in the same phenotypic outcome, i.e. loss of the midbrain-hindbrain boundary region.

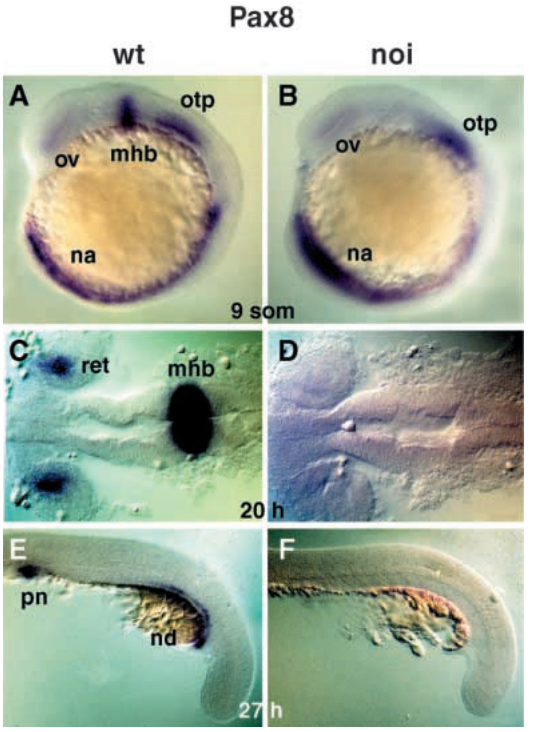


Fig. 9. Requirement of the *noi* (*Pax2.1*) function for *Pax8* expression. Wild-type (A,C,E) and noi^{tu29a} mutant (B,D,F) embryos were analyzed for *Pax8* expression at the 9-somite stage (lateral view) as well as at 20 hours (dorsal view) and 27 hours postfertilization (lateral view). For abbreviations see legend to Fig. 3.

The *Pax2.1* (*noi*) function is required for the maintenance of *Pax5* and *Pax8* transcription in other expression domains

The midbrain-hindbrain boundary region is the only tissue which is lost in *noi* mutant embryos until the pharyngula period (28 hours), as shown by the persistence of mutant *Pax2.1* mRNA in all other expression domains (Brand et al., 1996). Consistent with this observation, the *Pax2.2* gene is also normally expressed at 20 hours in the optic stalk (Fig. 7D) and in the otic vesicle, as well as in hindbrain and spinal cord neurons of *noi* mutant embryos (data not shown). In contrast, *Pax5* expression is lost in the otic vesicle (Fig. 8D). Moreover, *Pax8* transcripts are no longer observed at the pharyngula stage

in the retina (Fig. 9D), pronephros and nephric ducts of mutant embryos (Fig. 9F), although this gene is normally expressed at the 9-somite stage in the primordia of these structures (Fig. 9B). The loss of *Pax2.1* (*noi*) activity has, however, no effect on the expression of *Pax5* and *Pax8* in the hindbrain and spinal cord (Fig. 8B; data not shown). Hence, we conclude that the continued presence of functional Pax2.1 protein is required for maintaining *Pax5* and *Pax8* transcription in all expression domains other than the hindbrain and spinal cord.

DISCUSSION

Evolution of the vertebrate *Pax2/5/8* genes

Pax258 genes have recently been isolated from several invertebrates (Czerny et al., 1997; Fu and Noll, 1997), indicating that these species contain a single member of the *Pax2/5/8* family in their genome, in contrast to the three members present in higher vertebrates (Adams et al., 1992). Here we have demonstrated the presence of *Pax2*, *Pax5* and *Pax8* orthologues in two lower vertebrates, the zebrafish and pufferfish. Hence, the three members of the *Pax2/5/8* family must have arisen by gene duplications before the diversification of vertebrates. In contrast to mammals, we have identified a second zebrafish *Pax2* gene, referred to as *Pax2.2*, in addition to the known *Pax2.1* (*Pax[b]*) gene (Krauss et al., 1991). Several gene families, including the *hox*, *pou*, *otx*, *msx*, *dlx*, *brn*, *engrailed* and *hedgehog* genes, have been shown to contain additional members in zebrafish compared to mammals, thus indicating that the genome has undergone a partial duplication in the zebrafish lineage (Postlethwait et al., 1998, and references therein). Although the *Pax2.2* gene differs from *Pax2.1* at 48% of all silent sites, it still codes for a full-length Pax2 protein, thus demonstrating that natural selection has maintained *Pax2.2* as a functional gene ever since the relatively ancient gene duplication event. It is, however, unclear at present why fish, in contrast to mammals, have a second functional *Pax2* gene, particularly since *Pax2.1* closely resembles the mammalian *Pax2* gene both in its expression and function.

Different members of the zebrafish *Pax2/5/8* family are expressed in a spatially and temporally overlapping manner during the development of five distinct structures (Fig. 10), thus pointing to a primordial role of the ancestral *Pax258* gene in the morphogenesis of the isthmus, the hindbrain and spinal cord, the optic stalk, the otic region and the pronephric system. In addition, all four Pax2/5/8 proteins of the zebrafish have been highly conserved in functional domains such as the paired domain (DNA-binding), octapeptide motif (protein-protein interaction) and C-terminal regulatory module (transactivation). Hence, these transcription factors may fulfill similar biochemical and thus partially redundant functions, which is further supported by the fact that the mammalian Pax2/5/8 proteins bind to DNA and activate transcription in a very similar manner (Kozmik et al., 1993; Dörfler and Busslinger, 1996; Czerny et al., 1997). The overlapping expression patterns of the *Pax2/5/8* genes therefore suggest some functional redundancy of these genes in zebrafish development. Interestingly however, inactivation of the *Pax2.1* gene by the *noi* mutation leads to developmental abnormalities in four of its five expression domains, i.e. in the midbrain-hindbrain boundary region, pronephros, optic stalk and otic region (Brand et al., 1996; Macdonald et al., 1997; Lun and Brand, 1998). Expression analyses of the newly identified *Pax* genes in *noi* mutant embryos have now revealed a genetic hierarchy among the different members of the *Pax2/5/8* family, thus resulting in a reinterpretation of the *noi* phenotype and providing an explanation for the phenotypic differences of *Pax2*(*.1*) gene mutations in mouse and zebrafish. Below we will discuss the different aspects of the *noi* phenotype in light of the expression data of the newly identified zebrafish *Pax2/5/8* genes.

The role of *Pax* genes in the development of the midbrain-hindbrain boundary region

The most dramatic difference between the mouse *Pax2* and

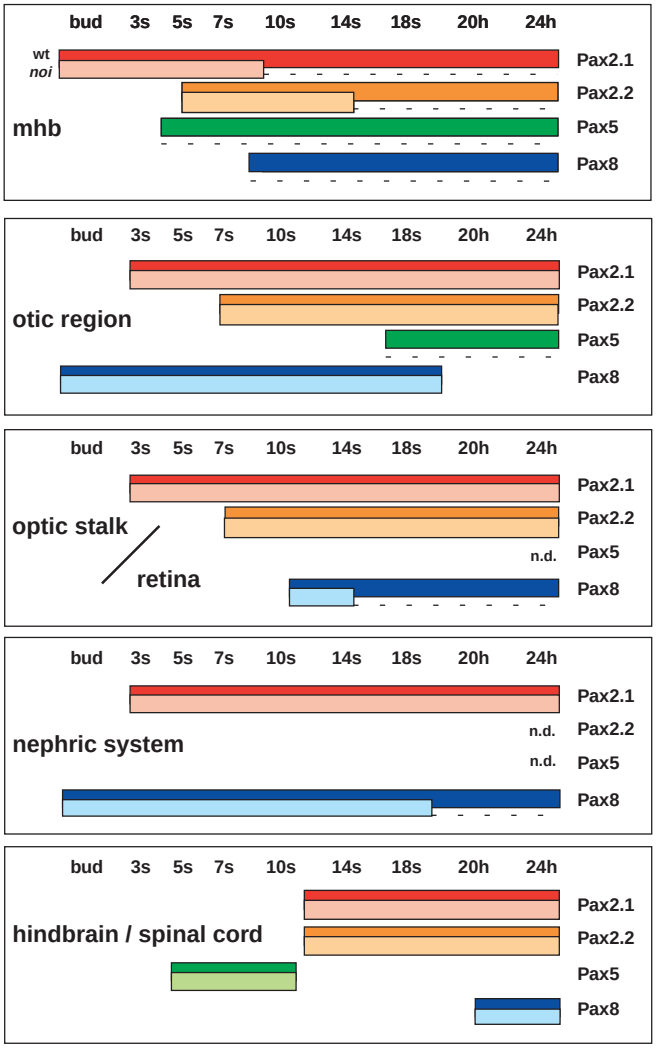


Fig. 10. Summary of the temporal regulation of all four *Pax2/5/8* genes in wild-type and *noi* mutant embryos of the zebrafish. The mRNA expression patterns of the different *Pax* genes are schematically shown, based on the results of whole-mount in situ hybridization analyses. No attempt has been made to quantitate the transcript levels. *Pax* gene expression in wild-type embryos (wt) is in dark colours, while light colours refer to the corresponding expression in *noi*^{tu29a} mutant embryos. Dashes symbolize the absence of *Pax* gene expression in mutant embryos. h, hours; s, somites; n.d., no expression detected.

zebrafish *noi* mutations is observed in the development of the midbrain and cerebellum, which is known to depend on an organizing centre located at the midbrain-hindbrain boundary. Genetic studies in the mouse revealed that the activity of this isthmic organizer requires expression of the secreted factors Wnt-1 and Fgf-8, as well as of the homeodomain transcription factors Otx-2, Gbx-2 and En-1 (reviewed by Wassef and Joyner, 1997). In zebrafish furthermore, the maintenance of the organizer is critically dependent on the *Pax2.1* (*noi*) function (Brand et al., 1996). In *noi* mutant embryos, the primordium of the midbrain-hindbrain boundary is initially formed, but is then lost during later development, due to misspecification and subsequent apoptosis of the entire region (Brand et al., 1996; Lun and Brand, 1998; Figs 7D, 9D). In contrast, inactivation of the mouse *Pax2* gene results in a highly variable, strain background-dependent phenotype, which ranges from frequent deletion (Favor et al., 1996) or exencephaly (Torres et al., 1996) to normal development of the midbrain-hindbrain boundary region (Schwarz et al., 1997). The gene compensating for the loss of Pax2 activity has recently been identified to be *Pax5*, whose targeted inactivation alone minimally affects the development of the isthmic region (Urbánek et al., 1994). However, consistent deletion of the posterior midbrain and cerebellum is observed in *Pax2*, *Pax5* double-mutant embryos, thus demonstrating dosage-dependent cooperation of both transcription factors in the development of the midbrain-hindbrain boundary region (Urbánek et al., 1997; Schwarz et al., 1997).

All members of the *Pax2/5/8* family are expressed at the midbrain-hindbrain junction in both zebrafish and mouse embryos, and even their onset of expression has been strictly conserved between the two species, with *Pax2*(*.1*) initiating before somitogenesis followed by *Pax5* at approx. 4 somites and *Pax8* at approx. 9 somites (for zebrafish see Fig. 10; for mouse see Urbánek et al., 1994; Rowitch and McMahon, 1995; Song et al., 1996; Fig. 6I). Interestingly however, the expression of these *Pax* genes radically differs in *Pax2*(*.1*) mutant embryos of the two species. Whereas transcription of *Pax5* is correctly activated at the midbrain-hindbrain boundary of mouse embryos even in the absence of *Pax2* function (Torres et al., 1996), initiation of *Pax5* and *Pax8* expression is never observed in the corresponding region of *noi* mutant zebrafish embryos (Fig. 10). Hence, the Pax2.1 (noi) protein is directly or indirectly involved in transcriptional activation of *Pax5* and *Pax8* at the midbrain-hindbrain junction, while no genetic evidence exists for a similar cross-regulation in the mouse. Although the expression of the zebrafish *Pax2.2* gene is independent of the *Pax2.1* (*noi*) function (Fig. 10), the Pax2.2 protein appears to be unable to compensate for the combined loss of Pax2.1, Pax5 and Pax8, possibly due to its low expression level and later onset at the midbrain-hindbrain boundary. Consequently, the development of the isthmic region cannot be maintained in *noi* mutant embryos. As the expression of *Pax5* is lost in these embryos, the *noi* mutation can be regarded as formally equivalent to double mutation of the mouse *Pax2* and *Pax5* genes with respect to the development of the midbrain-hindbrain boundary region. Hence, the distinct phenotypes of the *Pax2*(*.1*) mutations in zebrafish and mouse result from species-specific differences in the regulation of *Pax2/5/8* genes during brain development.

Pax genes and the development of the excretory system

Both the zebrafish *Pax2.1* and mouse *Pax2* genes are essential for the development of the excretory system, despite the fact that kidney organogenesis differs in several aspects between the two species. In mammals, three embryonic kidneys, the pronephros, mesonephros and metanephros, sequentially develop during embryogenesis, although only the metanephros ultimately gives rise to the adult kidney (Saxén, 1987). Inactivation of the mouse *Pax2* gene prevents formation of both the mesonephros and metanephros, while its effect on pronephros development has not yet been investigated (Torres et al., 1995; Favor et al., 1996). In fish, the pronephros matures into a functional excretory organ at the larval stages and is subsequently replaced in the adult fish by the mesonephric kidney, known as the opistonephros (Saxén, 1987). In *noi* mutant embryos, the pronephros and nephric ducts are initially formed, but are then lost during the late stages of the pharyngula period (Brand et al., 1996).

As in mammals, two *Pax* genes are expressed during kidney morphogenesis of zebrafish. In mouse embryos, *Pax2* and *Pax8* are already transcribed during pronephros development (Dressler et al., 1990; Fig. 6I). In zebrafish, the expression of *Pax8* even precedes that of the *Pax2.1* gene in the intermediate mesoderm, which later gives rise to the nephric organs (Fig. 10). Interestingly, the expression of the zebrafish *Pax8* gene is correctly initiated in the pronephric anlage of *noi* mutant embryos, but is then lost in the pronephros and nephric ducts as early as the 20-somite stage (Fig. 10). Hence, the maintenance of *Pax8* expression in the nephric system is dependent on the *Pax2.1* (*noi*) function, thus indicating a genetic interaction between the two genes. As the *noi*-independent *Pax2.2* gene is not even expressed during normal kidney development (Fig. 10), it follows that no protein of the Pax2/5/8 family is synthesized anymore past the 20-somite stage in the nephric system of *noi* mutant embryos. As a consequence, the entire excretory system is lost by 36 hours of development (Brand et al., 1996).

Expression of multiple *Pax2/5/8* genes in the developing eye and ear

Developmental abnormalities of the eye are a consistent feature of heterozygous *Pax2* mutations both in humans (Sanyanusin et al., 1995) and mice (Favor et al., 1996), indicating that the morphogenesis of this organ is highly sensitive to the *Pax2* gene dosage in mammals. The eye phenotype of homozygous *Pax2*(*.1*) mutations is similar in some aspects but discrepant in others between zebrafish (Macdonald et al., 1997) and mouse (Torres et al., 1996). In both species, the *Pax2(.1)* function is required for the closure of the optic fissure and for differentiation of the optic stalk into glial cells, which participate in myelination of optic nerve axons. The optic nerve furthermore exhibits pathfinding defects in both species, although they are different in nature. While most of the retinal axons in wild-type mouse embryos cross to the contralateral side of the brain at the optic chiasma, these axons never reach the midline and project ipsilaterally in *Pax2* mutant embryos, which lack an optic chiasma. In zebrafish, the optic chiasma is still formed even in the absence of *Pax2.1* (*noi*); yet navigational errors of retinal axons are still observed, although they vary considerably in individual embryos. This difference

and variability of the *noi* phenotype may indicate partial compensation of the loss of the *Pax2.1* (*noi*) function by *Pax2.2*, which is expessed in a *noi*-independent manner during optic stalk development. However, *Pax2.2* is unable to fully compensate, probably due to its delayed onset of expression relative to the *Pax2.1* gene (Fig. 10). Interestingly, even the *Pax8* gene is transcribed in the developing eye, but its expression cannot be maintained in *noi* mutant embryos (Fig. 10).

Pax2 is also essential for normal morphogenesis of the cochlea and spiral ganglion in the inner ear of the mouse embryo (Favor et al., 1996; Torres et al., 1996). While a detailed characterization of the corresponding *noi* phenotype has not yet been reported in zebrafish, a slight reduction in the size of the otic vesicle has been noted (Brand et al., 1996). Interestingly, all members of the zebrafish *Pax2/5/8* family are expressed during development of the otic system (Fig. 10). Moreover, the expression of *Pax5* is lost in the otic vesicle of *noi* mutant embryos, which also implicates *Pax2.1* in the regulation of *Pax5* during ear development (Fig. 10). Importantly, *Pax8* is transiently expressed in the otic region even before *Pax2*(*.1*), both during zebrafish and mouse embryogenesis, thus implying that *Pax8* is the earliest marker of the vertebrate otic anlage.

Cross-regulation of *Pax* genes

The most intriguing finding of our study is the dependency of *Pax5* and *Pax8* expression on the *Pax2.1* (*noi*) function during zebrafish development. Similar observations have been reported for *Pax* genes of *Drosophila*, suggesting that cross-regulatory interactions may be an ancient feature of *Pax* gene duplications (Czerny et al., 1997, and references therein). In particular, the *twin-of-eyeless* (*toy*) gene of *Drosophila* has recently been shown to act upstream of *eyeless* (*ey*) in the eye morphogenetic pathway (T. Czerny, G. Halder, W. Gehring and M. Busslinger, unpublished data). The intercalation of one of the duplicated *Pax* genes below the other in the same developmental pathway could best be explained by assuming that the ancestral gene may have been under autoregulatory control. Following duplication, both genes would initially have had the potential to cross-regulate each other. However, the subsequent loss of regulatory elements in one gene may have led to the establishment of a hierarchical relationship between the duplicated genes. As predicted by this hypothesis, the Toy protein of *Drosophila* has recently been shown to directly interact with the eye-specific enhancer of the *ey* gene (Czerny et al., 1998). By analogy, it will be of interest to see whether the Pax2(.1) protein also directly regulates *Pax5* and *Pax8* by binding to their control regions.

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